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Functional Characteristics of Tumor-Associated Protein Spot14 and Interacting Proteins in Mouse Mammary Epithelial and Breast Cancer Cell Lines

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13. SUPPLEMENTARY NOTES

14. ABSTRACT Thyroid Hormone Responsive Protein Spot14 (S14) is known to be necessary for high rate de novo fatty acid synthesis, and elevated S14 is correlated with reduced disease free survival of women afflicted with breast cancer. The molecular mechanism of S14 remains illusive. Two models exist for S14 function: one implicated with transcriptional events and the other in metabolic processes. These findings suggest that S14 may not directly influence lipogenic gene expression. To that end, exogenous S14 fused to a nuclear localization sequence (NLS) trafficed to the nucleus but did not alter S14 responsive genes ME1 and PCx. Despite the shift toward lipogenesis indicated by Bodipy and NMR, exogenous S14 did not alter glycolytic or lipogenic enzyme levels in normal or ErbB2 tumor cells. A major finding in this report is that glycolytic and lipogenic enzyme abundance was altered in a serum dependent manner for normal but not ErbB2 cells. S14 overexpression in normal CiT3 cells, but in not ErbB2 cells, provided a growth advantage when serum was depleted of lipid and had low glucose. ErbB2 cells did not respond to progestin regardless of S14 overexpression because they lack the progesterone receptor. Together, these results show that S14 does not alter gene expression in these cell culture systems.

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Introduction

Cancer cell metabolism differs substantially from quiescent, normal cell metabolism [1]. While regulation of anabolic metabolism (i.e. lipid biosynthesis) is important to the normal growth of mammary epithelial cells, breast cancers are often characterized by elevated fatty acid synthesis [2], and those increases correlate with reduced disease free survival of breast cancer afflicted women [3]. Two prognostic indicators of aggressive breast tumors are fatty acid synthase (FASN) and thyroid hormone responsive protein Spot14 (S14) [4]. Much is understood regarding regulation of FASN, but little is known about the regulation of S14 or its molecular mechanism. When S14 is lost due to genomic knock out in mice [5] or siRNA knockdown in hepatocyte cell culture [6], a reduction of de novo fatty acid synthesis follows. Two lines of evidence exist for S14 function in modification of metabolism: one in the nucleus to regulate transcriptional/mRNA processes [7], the other in the cytosol at the protein level to alter lipogenic enzyme activity [8]. This study attempts to understand what hormones regulate S14 gene expression, and to identify potential S14 interacting proteins that confer its function.

Review

The broad goals are to examine the functional characteristics of tumor associated S14 and to identify potential interacting proteins to elucidate mechanism. The level of endogenous S14 gene expression and protein abundance is miniscule in both normal CiT3 mammary cells and ErbB2 mammary tumor tissue culture cells. This finding prompted generation of stable, doxycycline-inducible S14 normal CiT3 and ErbB2 tumor cell lines. Normal mammary epithelial cells under growth conditions, that stably overexpress doxycycline inducible S14, have only subtle differences in expression of glycolytic and de novo fatty acid genes. Only the chief glucose transporter (Glut1), aldolase C and pyruvate carboxylase had significantly different levels under growth conditions. Although statistically significant for change among biological replicate groups, the changes were subtle (< 2-fold) and possibly not relevant to the biology. In addition to gene expression profiling, protein abundance was evaluated by immunoblot. Overexpression of S14 under either growth or differentiation conditions did not vary protein levels of glycolysis and lipogenic pathway enzymes noticeably. In contrast to S14 differences, comparison between growth and differentiation conditions revealed prolactin dependent changes in gene and protein abundance.

While stable overexpression of S14 had minor influences on gene expression but not on protein abundance, neutral lipid staining showed that CiT3 cells overexpressing S14 store more lipid than controls regardless of growth or differentiation conditions. Further analysis of the lipid component using NMR Metabolomics showed significant increases in the quantity of intracellular $(CH2)_n$ and (CH3) acyl chains (i.e. fatty acids). Together, these data suggest that S14 activity occurs not at the transcriptional level to directly influence gene expression, but at the level of enzyme activity to shifts cell biology toward anabolic metabolism. Therefore, I hypothesize that S14 interacts with metabolic enzymes to carry out its function. In order to identify potential interacting proteins, a co-immunoprecipitation (co-IP) and mass spectrometry approach was used. Initial screens of the co-IP's identified a handful of metabolic proteins that could associate with S14 including phosphofructokinase 1, lactate dehydrogenase, pyruvate kinase M2, and aldoase A, but follow up immunoblots for S14 associated proteins have failed. Also, any interaction with de novo fatty acid synthesis pathway enzymes was also not observed, likely due to the type of serum used in the medium.

Large amounts of evidence is being gathered to suggest that mouse S14 does not act to regulate transcriptional activation, as has been shown in rat and human cell lines. Still, much of this evidence remains purely correlative; and as of the time of this report, no direct causal link has been made to alterations in metabolism or metabolic gene expression with the overexpression of S14. Many of these experiments were conducted in normal mouse mammary cells, and now attention is shifting to the ErbB2 tumor cell models to investigate S14 action.

Body

Nuclear S14 does not induce target gene expression

Last year's data raised questions regarding the conflicting paradigms for S14 function. The function of tumor-associated protein S14 has long been associated with the synthesis of fatty acids de novo, but little is

understood about S14 mechanism. The conflicting paradigms regarding S14 mechanism include one involved with direct modification to transcription events in the nucleus [4, 6, 9]. The possibility that S14 traffics to the nucleus to modulate gene expression was recently reported [7]. The report from Chou et al. showed that a GFP-S14 fusion protein migrated to the nucleus to affect p53 dependent target genes. It should be noted that endogenous mouse S14, *in vivo*, has not been detected in the nucleus using multiple methods, such as immunofluorescence (illustrated in Figure 1) and sub cellular fractionation (data not shown). While evidence for S14 nuclear localization has been reported in hepatocytes, it is possible that the protein behaves differently in mammary cells. I considered the possibility that S14 may significantly alter gene expression in the mammary gland or breast tumor if it could be localized to the nucleus. To explore this phenomenon in normal and tumor cell lines, I constructed a series of fusion proteins; first, one that consists of an N-terminal NLS (nuclear localization sequence) from SV40 that traffics into the nucleus; second, an N-terminal FLAG-S14 construct was constructed to verify cytosolic localization and to confirm that the C-terminal HA tag did not obfuscate any potential nuclear S14 activity. The NLS-S14 was evaluated for cellular localization using two independent methods to determine if the NLS indeed drives S14 into the nuclear compartment. Likewise, the FLAG construct should be restricted to the cytosol, even though small proteins, such as S14, can readily migrate into the nucleus.

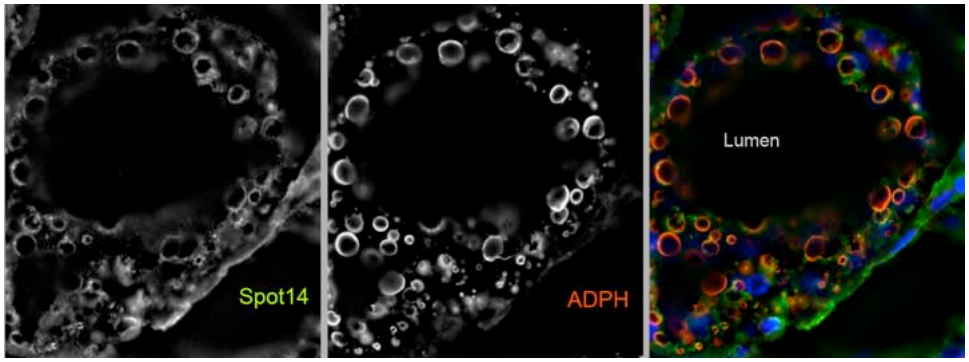

Figure1. Localization of mouse S14 in lactation day 6 mouse mammary gland. S14 shows cytosolic staining (green) but not nuclear staining as indicated by DAPI (blue). ADPH (adipophilin, red) is shown as a mammary epithelial specific cytosolic marker known to coat cytosolic triglyceride droplets.

First, immunofluorescence was used to qualitatively assess the localization of NLS-S14 and FLAG-S14 following transient transfection into normal CiT3 cells. In Figure 2, S14 is green, red is phalloidin-stained actin, blue is DAPI stained DNA. NLS-S14 shows nuclear localization as cyan coloring confined to the DAPI stained nuclear regions, with sparse amounts observed in the cytosol. Conversely, the N-terminal FLAG tagged construct showed no nuclear localization (similar to figure 1). Cytosolic localization was also confirmed for the S14-HA dox inducible CiT3 cells (data not shown). Using immunofluorescence, only the S14 that contained the NLS was observed retained in the nucleus, while FLAG-S14, S14-HA, and endogenous S14 appear to be confined to the cytosol.

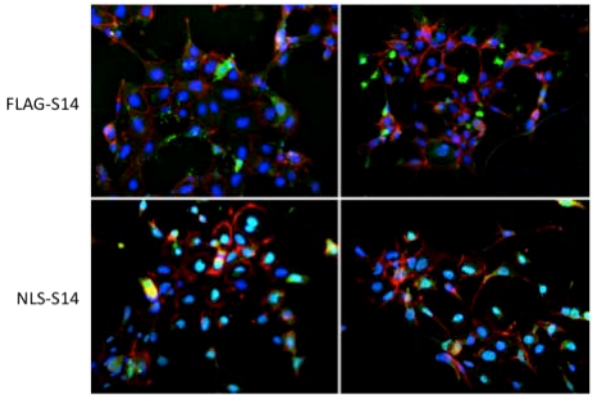

Figure 2. FLAG and NLS tagged mouse S14 transient transfection in normal CiT3 cells. Only the NLS construct is localized to the nucleus, whereas the N-terminal Flag construct is confined to the cytosol.

The second method used to evaluate localization of the NLS construct was sub-cellular fractionation, which separates the cytosolic and nuclear compartments. Figure 3 shows the cellular compartmentalization of the various S14 constructs, including N-terminal FLAG-S14, N-terminal NES-S14 (Nuclear Export Sequence, this construct failed), N-terminal NLS-S14, and the C-terminal S14-HA. The NLS construct again targets to the nucleus (anti-mouse S14), while a

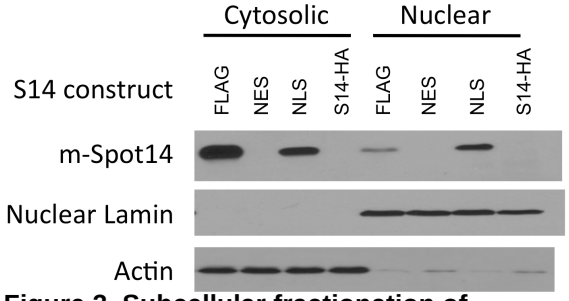

Figure 3. Subcellular fractionation of cytosylic and nuclear compartments in normal CiT3 cells. FLAG-S14 is confined to the cytosolic fraction, and NLS-S14 localizes to the nuclear fraction. Nuclear lamin is shown as a compartment specific loading control.

small amount of FLAG-S14 is also detected in the nuclear fraction (Lamin as a nuclear loading control, actin as a cytosolic loading control). The FLAG-S14 in the nuclear fraction is likely cytosolic 'contamination,' as small amounts of actin were also detected in this fraction. Combined with the immunofluorescence data, the fractionation data confirm S14 can be driven into the nucleus.

In order to assess if nuclear localized S14 is competent to modify gene expression, NLS-S14 and FLAG-S14 were transiently transfected into normal CiT3 cells under growth conditions (Figure 4). Cit3-S14HA cells were also examined for these three target genes as a control. To begin evaluating the effect of S14 on lipogenic gene expression, I interrogated the mRNA levels of three genes shown to be *induced* by S14 in other rat hepatocytes. While not remotely exhaustive, these three proposed S14 target genes, Fasn, Me1, and Pcx, did not increase in expression 48 hours after S14 construct transfection in either the CiT3 cells, or following dox-mediated induction of S14 in CiT3-S14HA cells. Thus, nuclear localized S14 did not act as a transcriptional activator of three genes reported to be affected by S14 in hepatocytes. However, the NLS-S14 construct still needs to be evaluated for nuclear S14 influence in ErbB2 tumor cells, as tumor cells could respond differently.

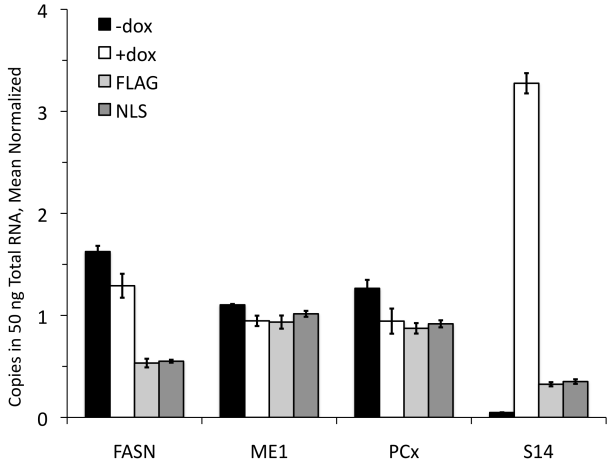

Figure 4. Transient transfection of NLS-S14 and FLAG-S14 constructs into CiT3-S14HA cells. qPCR gene expression shows copy number for potential S14 target genes in 50ng of total RNA normalized the mean. No significant expression changes were observed.

Mouse S14 Homodimerizes

Early yeast two hybrid (rat S14) and cell free binding studies of human S14 showed that S14 interacts with itself [10], but no evidence shows that this interaction occurs in mammary cells. Several experiments now demonstrate that mouse S14 forms homodimers. First, recombinant mouse S14 (m-S14) was expressed in BL21 bacteria, poly-histidine nickel column purified, thrombin cleaved, and subjected to analytical size exclusion chromatography. At 300 µM concentration, m-S14 showed a single elution run time that was consistent with a protein of approximately 37 kDa (recombinant m-S14 monomers are predicted at approximately 18 kDa), suggesting that m-S14 is solely a dimer in solution. The same result was returned for 30 µM, indicating that the Kd for binding is at least below this concentration.

In order to address if m-S14 can self-associate within mammary cells, FLAG-S14 was transiently transfected into the dox inducible CiT3-S14HA cells. In triplicate, transfected cells were either induced or not with 0.2 µg/mL doxycycline for 48 hours. Cells were lysted in non-denaturing buffer, and immunoprecipitation was carried out with either anti-HA agarose or anti-FLAG agarose beads (figure 5). The left panel clearly shows the co-IP of the FLAG construct when S14-HA is pulled down. The reciprocal IP is less robust, probably due to more modest induction of the transgene using 0.2 µg/mL doxycycline and overwhelming FLAG-S14. To my knowledge, this is the first evidence for mouse S14 that shows self-association in the context of the mammary epithelial cell, which corroborates previous reports that show rat S14 homodimerizes.

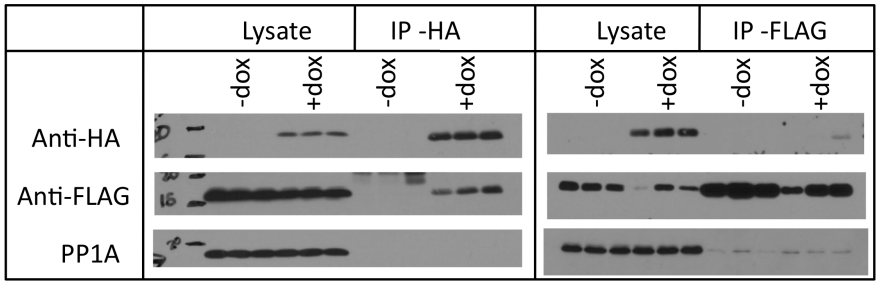

Figure 5. Co-immunoprecipitation of FLAG-S14 with S14-HA in normal mouse mammary epithelial cells. Cit3-S14HA cells were transiently transfected with the FLAG-S14 construct. Samples were immunoprecipitated with either anti-HA or anti-FLAG agarose beads. Immunoblots for the HA or the FLAG tag show that mouse S14 can self associate in CiT3 cells.

S14 Overexpression has minor effects on Glycolysis and Lipogenic Pathways

One key to understanding the metabolic influence of S14 in the lipogenic pathway is to be able to alter the cell's use of the pathway by changing the available nutrients. The objective of the following experiments was to establish a model with which to test the effect of S14 on lipid biosynthetic enzymes. A major finding of this study is that the enzymes that support de novo fatty acid synthesis are affected by the concentration of serum triglyceride and cholesterol. In tissues that actively synthesize fatty acids, such as liver and adipose, regulation of the lipogenic pathway is known to require SREBP transcription factors [11]. In these tissues, the membrane concentration of intracellular cholesterol is sensed and maintained with exquisite precision [12]. Altogether, when ER membrane cholesterol levels drop, SREBP transcription factors are activated to induce the pathway genes that code for fatty acid and cholesterol biosynthesis. I hypothesized that regulation of the de novo fatty acid synthesis pathway was also regulated by this mechanism in normal mammary epithelial cells.

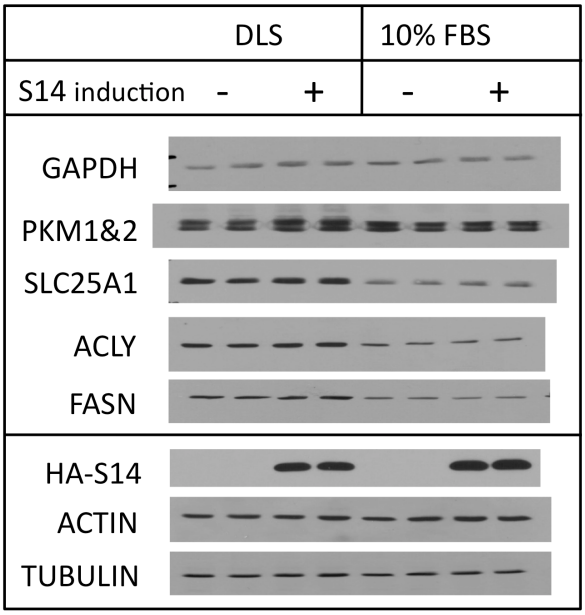

Figure 6. Effect of Lipid Reduced Serum and 10% FBS on glycolytic and lipogenic enzyme levels in CiT3-S14HA cells. CiT3-S14HA cells were cultured in either DLS serum lacking cholesterol and TAG or 10% FBS replete with cholesterol and TAG. Immunoblots show that lipogenic proteins SLC25a1, ACLY, and FASN are elevated by DLS conditions.

Figure 6 shows the effect in Cit3-S14HA cells cultured for 72 hours in serum depleted of TAG and cholesterol (DLS) or in medium with 10% fetal bovine serum that is replete with TAG and cholesterol. Two enzymes in the glycolysis pathway (GAPDH and PKM1&2) are not affected by serum treatment and are also not influenced by S14 overexpression. In contrast, three proteins that constitute a linear series of reactions from citrate to fatty acid, SLC25a1 (the citrate transporter), ACLY (that converts citrate into acetyl-CoA), and FASN (that synthesizes fatty acids from acetyl-CoA), are all induced when serum cholesterol and TAG are limited. Interestingly, the protein levels are not altered in a S14 dependent manner, regardless of the serum used. These data contradict a 1997 report by Brown et al. showing knockdown of S14 in primary hepatocytes diminished levels of ACLY and FASN protein [6].

Importantly, figure 6 demonstrates that three known targets of SREBP regulation, SLC25a1 [13], ACLY and FASN [11] are all upregulated under DLS conditions. Previous transduction of CiT3 cells with activated SREBP1c adenovirus induced FASN nearly 8-fold after 24 hours (data not shown). If the lack of cholesterol in the culture medium activates SREBP transcription factors, then gene expression of SLC25a1, ACLY and FASN should also be upregulated. Surprisingly, the level gene expression for these known SREBP targets remained relatively unchanged (black versus charcoal bars). Additionally, there was no influence of S14 overexpression upon the levels of these genes. This result suggests that SREBP1 is not activated by depleted serum cholesterol levels in this cell type; and, because gene expression is unchanged, some form of post-transcriptional regulation of SLC25a1, ACLY and FASN exists in normal mammary epithelial cells. Further, minor changes in gene

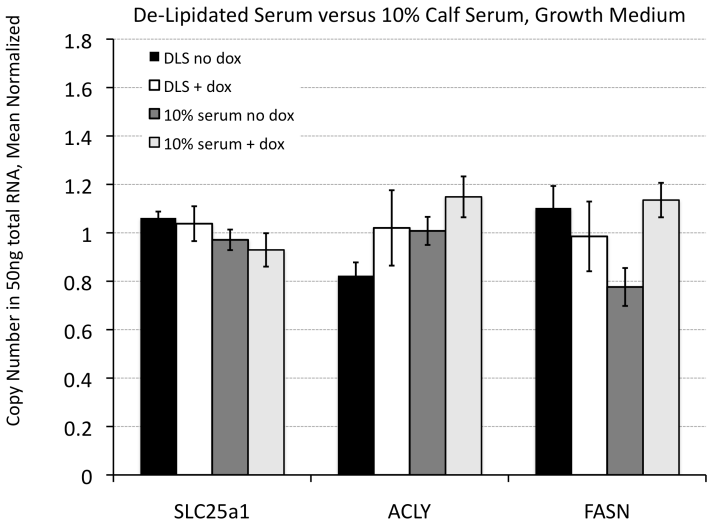

Figure 7. Effect of DLS and 10% BCS on SREBP target gene expression. CiT3-S14HA cells were cultured in DLS or BCS in the presence or absence of S14 overexpression. Gene expression of three SREBP targets was not altered in cholesterol limited DLS serum, suggesting alternative regulation of this de novo fatty acid synthesis pathway.

expression of glycolytic enzymes ALDOc, GAPDH, and Malic Enzyme were observed, but it is not clear if these are SREBP targets. It will be necessary to measure the levels of SREBP2 target genes, because those are exclusively involved in cholesterol biosynthesis [12].

Tumor cell metabolism is often refractory to nutrient signals, such as lipids, that otherwise suppress metabolic proteins in normal cells [14, 15]. Using a similar approach to the DLS studies in CiT3-S14HA cells, I tested whether serum lipids in the medium influence ErbB2 mammary tumor cells. In order to control for the types of lipid presented to the cells, 19 µg/mL oleic acid (OA) was supplemented in 2% DLS, and the glucose was restricted to physiological 5.5 mM. Figure 8 shows an immunoblot for several enzymes of the de novo fatty acid synthesis pathway in the presence or absence of S14 overexpression and varied serum conditions. Interestingly, the lipid-depleted serum does not affect the abundance of enzymes in the de novo fatty acid pathway in the 78617 ErbB2 tumor cells. The citrate transporter, SLC25a1, phosphorylated (active) and total ACLY, and total ACC1 do not vary. In contrast, phosphorylated ACC1 (inhibited) is elevated relative to 2% DLS, indicating some inhibitory feedback for the de novo fatty acid pathway. This experiment is promising and should be repeated using 10% FBS to confirm that protein levels and gene expression in 78617 ErbB2 tumor cells respond differently than normal mammary cells when serum lipid is limited.

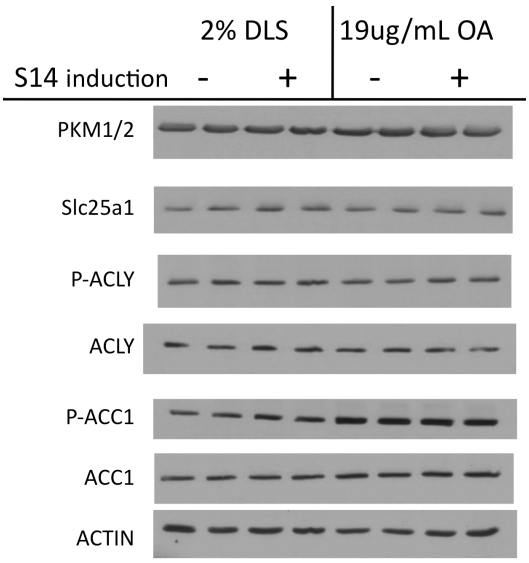

Figure 8. Protein levels for the de novo fatty acid synthesis pathway in 78617 ErbB2 tumor cells. 2% DLS or 2% DLS supplemented with 19 ug/mL oleic acid with physiologic 5.5 mM glucose in the presence or absence of S14 overexpression. Only phosphorylated (inhibited) ACC1 is different between serum conditions.

In order to examine the serum influence on protein levels in CiT3-S14HA cells, they were grown under identical conditions as in the 78617 studies. The level of phosphorylated (inhibited) ACC1 is unchanged in the CiT3-S14HA cells unlike in the ErbB2 tumor line. Conversely, phosphorylated (active) ACLY is starkly elevated in the 19 ug/mL 2% DLS conditions; and additionally, p-ACLY seems reciprocally regulated in a S14 dependent manner. In the 2% DLS, + S14, p-ACLY is lower than the non-induced samples, however, in the 2% DLS, 19 ug/mL OA the level of p-ACLY is greater than the non-induced samples. Not only does this result show that tumor biology responds differently to serum lipid conditions than do normal cells, but it also implicates ACLY as a possible de novo fatty acid synthesis enzyme that is a S14 modified protein. Use of these serum conditions on CiT3-S14HA cells may facilitate co-IP studies to identify ACLY (or others) as the first S14 binding partner.

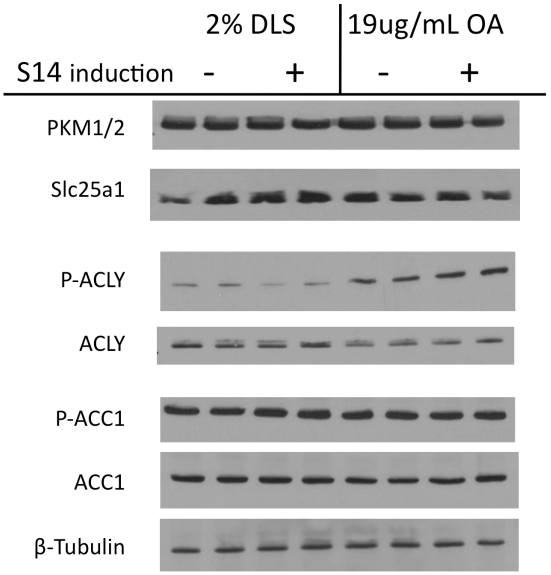

Figure 9. Protein levels for the de novo fatty acid synthesis pathway in CiT3-S14HA cells. 2% DLS or 2% DLS supplemented with 19 ug/mL oleic acid with physiologic 5.5 mM glucose in the presence or absence of S14 overexpression. Only phosphorylated (activated) ACLY changes between serum conditions, and in a S14 dependent manner.

S14 over expression confers a growth advantage in CiT3-S14HA cells but not ErbB2 tumor cells

Although the mechanism of S14 function remains illusive, it stands to reason that elevated synthesis of fatty acids could allow cells to also proliferate at a greater rate. Using the above strategy of limited serum lipids in DLS and under physiological 5.5 mM glucose conditions in the medium, I tested the hypothesis that overexpression of S14 would endow these cells better growth. ErbB2 tumor cells grew poorly when both glucose and serum were reduced, regardless of whether S14 was overexpressed (Figure 10). However, under these conditions the CiT3-S14HA cells had stunted proliferation without S14, but the S14 overexpressing cells proliferated more rapidly. Presumably the

normal CiT3-S14HA cells can adapt their metabolism to cope with both reduced serum lipids and low glucose levels, which the ErbB2 tumor cells are unable to do effectively.

ErbB2 tumor cells are non-responsive to progestin in Luciferase Reporter Assays

Part of Aim 1 of this project is to determine what hormones activate the human S14 luciferase promoter construct (huS14-Luc). This portion of the Aim has been addressed using the synthetic progestin R5020 that activates the progesterone receptor (PR) in HC-11 normal mouse mammary epithelial cells, T47D human breast cancer cells, and mouse ErbB2 tumor cells (Figure 11). All cells were grown in their normal respective media, and each sample was transiently transfected with 1 ug of human S14 luciferase promoter construct (kindly provided by Dr. Mariash) per well of a 12-well plate using lipofectamine 2000 according to the manufacturer's protocol. Only the HC11-PRb and T47D cells, which express PR, showed positive luciferase activity when stimulated with progestin. Both ErbB2 tumor cell lines did not respond to progestin, because they lack PR (data not shown).

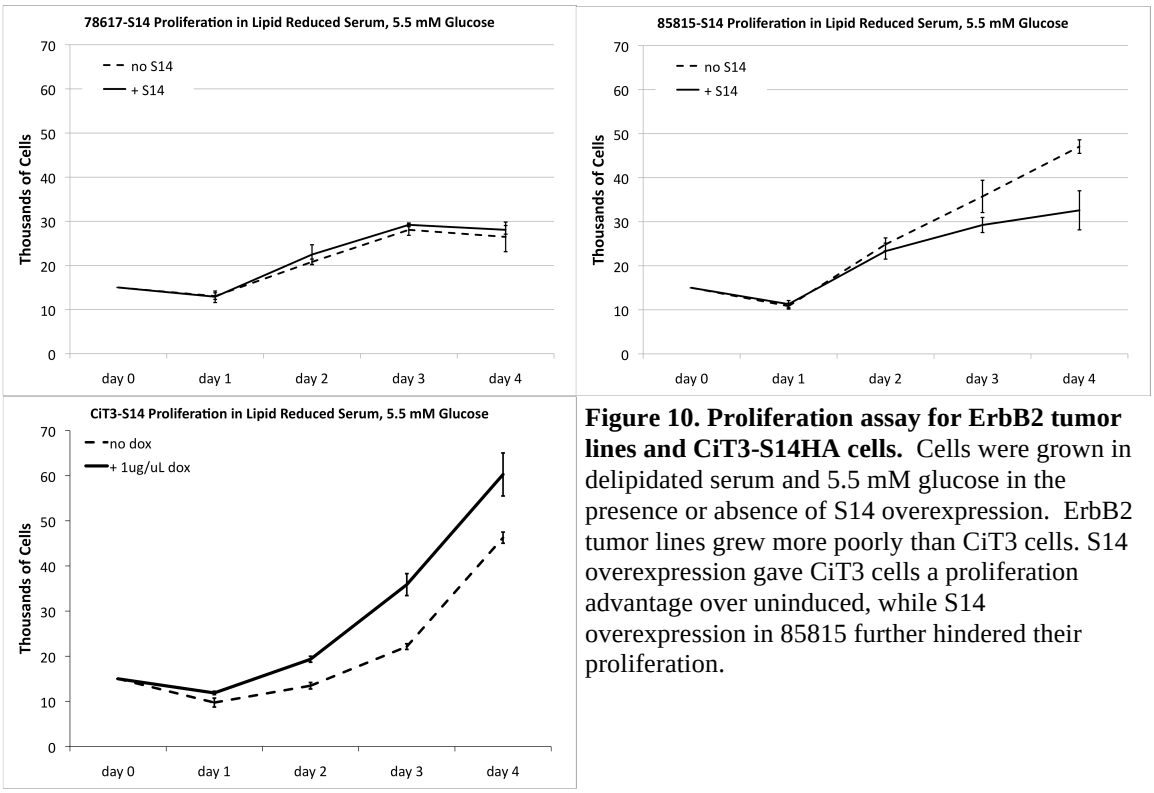

Figure 10. Proliferation assay for ErbB2 tumor lines and CiT3-S14HA cells. Cells were grown in delipidated serum and 5.5 mM glucose in the presence or absence of S14 overexpression. ErbB2 tumor lines grew more poorly than CiT3 cells. S14 overexpression gave CiT3 cells a proliferation advantage over uninduced, while S14 overexpression in 85815 further hindered their proliferation.

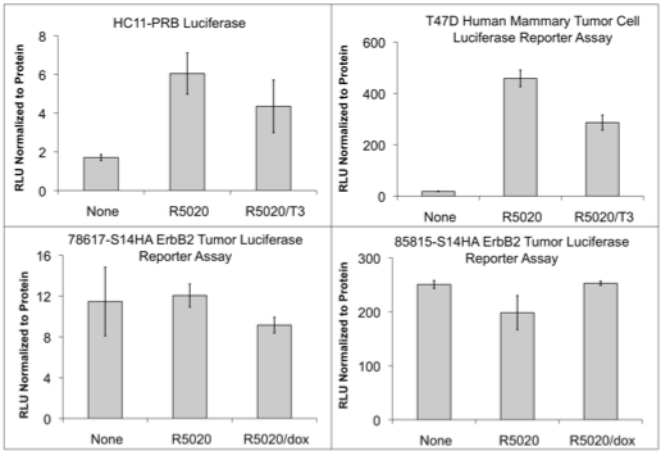

Figure 11. Human S14 luciferease reporter assay in human and mouse normal mammary and tumor cell lines. HC11-PRb normal mouse mammary cells that stably express human PRb (upper left), T47D human PR positive tumor cells (upper right), and ERbB2 mouse mammary tumor cells (lower panels) were transfected with a human S14 luciferase promoter construct. Cells were treated with R5020 or R5020 + T3 (upper panels) or R5020 or R5020 + doxycycline (lower panels). HC11 and T47D cells that express PR both respond to R5020. ErbB2 cells that lack PR show no response.

Key Research Accomplishments

1. S14-HA overexpression did not induce gene expression in CiT3-S14HA cells.
2. NLS-S14 localized to the nucleus, verified using two independent methods.
3. NLS-S14 driven to the nucleus did not induce expression of S14 target genes in CiT3 cells.
4. FLAG-S14 did not induce expression of S14 target genes in CiT3 cells.
5. S14 self associates, as determined by two independent methods; the first report that mouse S14 homodimerizes.
6. The de novo fatty acid synthesis pathway is stabilized when serum lipids are limited in normal CiT3 cells, but that ErbB2 tumor cells did not respond to lipid depleted serum.
7. S14 overexpression conferred a growth advantage when serum lipids were limited only in CiT3-S14HA cells, but not in either 78617-S14HA or 85815-S14HA ErbB2 tumor cells.
8. ErbB2-S14HA tumor cells do not respond to progestin R5020 because they do not express PR.

Reportable Outcomes

1. Data were presented at the Molecular Biology Program seminar (October 2009)
2. Data were presented at the Molecular Biology Program retreat (November 2009)
3. Data were presented at the Mammary Gland Program Project Retreat (January 2010)
4. Data were presented in the Pathology Research in Progress Seminar (March 2010)
5. Data were presented at the Breast Cancer Group Seminar (April 2010)

Key Training Accomplishments

1. Learned to perform co-immunoprecipitation techniques
2. Use of contemporary linear ion trap liquid chromatography (LTQ-LC) mass spectrometer (Agilent) for identification of co-IP proteins
3. Learned proteomics software, MASCOT and SCAFFOLD to identify peptide fragments and compare replicate data
4. Learned to perform cell proliferation assays
5. Learned to perform Luciferase reporter assays
6. Capability to measure gene expression at the copy number level
7. Use of state of the art Applied Biosystems 7500 Fast theromocycler for quantitative real-time PCR data acquisition
8. Learned to perform immunofluorescence on paraffin embedded tissue and fixed cell culture samples
9. Use of state of the art Olympus IX81 inverted motorized microscope with spinning disk attachment for deconvolution fluorescent images
10. Learned to extract aqueous and lipid metabolites from cells for NMR metabolomic analysis
11. One- and two-dimensional 1H-MR spectra were obtained using a Bruker 500 MHz DRX spectrometer (Bruker Bisopin, Fremont, CA) using an inverse TXI probe. For metabolite identification in water soluble and lipid mammary gland extracts, a two-dimensional (2D)-H, C-HSQC (heteronuclear single quantum correlation) technique was used

Conclusions

The function of tumor-associated protein S14 has long been associated with the synthesis of fatty acids de novo, but little is understood about S14 mechanism. Conflicting paradigms regarding the mechanism of S14 exist to induce lipogenic metabolism; one that suggests modification to transcription events in the nucleus [4] and the other suggests S14 works with metabolic proteins [8]. Because of this duality, a construct was generated to drive S14 into the nucleus (NLS-S14) to test whether S14 could induce its reported target genes. The attempt to drive S14 into the nucleus was successful, however, S14 induction of target genes was not observed suggesting that S14 behaves differently in normal mammary and mammary tumor cells with respect to hepatocytes. These results continue to support the hypothesis that S14 activity in mammary cells does not

function at the transcriptional level. The NLS experiments will be extended to the ErbB2-S14HA tumor lines to verify if nuclear S14 affects gene expression in these cells.

The progress outlined in this and the previous report demonstrates that overexpression of S14HA did not affect gene expression of the metabolic mRNAs interrogated, but rather S14HA appears to function at the cytoskeletal/enzyme level in normal mouse mammary cells based upon initial co-IP mass spec results. This effect was to be confirmed in the ErbB2 tumor cell lines, however, co-IP protein identification attempts with the ErbB2-S14HA tumor lines were not successful to date. The knowledge gained using culture medium with altered nutrient levels may prove to be the key to successful identification of S14 binding partners in either CiT3-S14HA or the ErbB2-S14HA cells.

S14HA overexpression shifts CiT3-S14HA cells towards anabolic metabolism based on Nile Red/Bodipy cytoplasmic lipid droplet staining and NMR metabolomics. Future studies will focus on S14HA overexpression in the ErbB2 tumor cell lines to determine if these trends are unique to the normal mammary epithelial cell metabolism. Due to difficulty and duration of running NMR experiments, use of 14C-glucose incorporation into fatty acids will be used. This approach will permit more rapid screening of cell lines and conditions at the expense of far less informative data.

Considering that activation/inhibition of the de novo fatty acid synthesis pathway enzymes differs between normal CiT3 and ErbB2 tumor lines (immunoblots shown here), overexpression of S14 in the ErbB2 cells may not yield informative results. Moreover, binding partners for S14 may not be expressed in these tissue culture cells. It is interesting to note that the level of endogenous S14 is very low in gene expression and not detectable at the protein level in all cultured cells examined. This observation may suggest that 2-dimensional cell culture systems cannot effectively model the biology of the S14 affected cancer, which naturally has elevated levels of tumor associated S14.

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